United States
Environmental Protection
Agency

Office of Water
Washington, D.C. 20460

EPA 821-R-00-026
September 2000

EPA

Method 1605: *Aeromonas* in Finished Water by Membrane Filtration

September 2000 - Draft

OCLC # 49863225

Acknowledgments

This method was prepared under the direction of Mary Ann Feige of the Office of Ground Water and Drinking Water's Technical Support Center within the U.S. Environmental Protection Agency's (EPA's) Office of Water (OW). This document was prepared under EPA OW Engineering and Analysis Division by DynCorp Information & Enterprise Technology, Inc.

Disclaimer

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Introduction

Aeromonas is a common genus of bacteria indigenous to surface waters, and may be found in non-chlorinated or low-flow parts of chlorinated water distribution systems. Monitoring their presence in distribution systems is desirable because some aeromonads may be pathogenic and pose a potential human health risk. Method 1605 describes a performance-based membrane filtration technique for the detection and enumeration of *Aeromonas* species. This method uses a selective medium that partially inhibits the growth of non-target bacterial species while allowing *Aeromonas* to grow. *Aeromonas* is presumptively identified by the production of acid from dextrin fermentation and the presence of yellow colonies on ampicillin-dextrin agar (ADA) medium. Yellow colonies are counted and confirmed by testing for the presence of cytochrome *c* (oxidase test) and the ability to ferment trehalose.

This method is for use in the Environmental Protection Agency's (EPA's) data gathering and monitoring programs under the Safe Drinking Water Act.

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Note: *Prior to validation of this method, each step of this method must be performed as written. Once the method is validated, this method will be performance-based. At that point, the laboratory is permitted to modify or omit any steps or procedure, provided that all performance requirements set forth in the validated method are met. The laboratory may not omit any quality control analyses. The terms "shall," "must," and "may not" indicate steps and procedures required for producing reliable results. The terms "should" and "may" indicate optional steps that may be modified or omitted if the laboratory can demonstrate that the modified, validated method produces results equivalent or superior to results produced by this method.*

Note: *This method has been revised based on reviewer comments on the April 2000 draft of this method.*

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Method 1605: *Aeromonas* in Finished Water by Membrane Filtration

September 2000 - Draft

1.0 Scope and Application

1.1 This method describes a membrane filter (MF) procedure for the detection and enumeration of *Aeromonas* in finished water samples. *Aeromonas* is a common genus of bacteria indigenous to surface waters. Its numbers are more likely to be greater during periods of warmer weather and when increased concentrations of organic nutrients are present. It also is more likely to be found in non-chlorinated, or low-flow parts of chlorinated water distribution systems. Some *Aeromonas* species are opportunistic pathogens.

1.2 This method is adapted from Havelaar et al. (1987) for the enumeration of *Aeromonas* species in finished water by membrane filtration (Reference 15.1), and has been briefly described as a proposed method in *Standard Methods for the Examination of Water and Wastewater, 20th edition,* Section 9260 (Reference 15.2). It is a quantitative assay that uses a selective medium which partially inhibits the growth of non-target bacterial species while allowing *Aeromonas* to grow. *Aeromonas* is presumptively identified by the production of acid from dextrin fermentation and bright yellow colonies that are greater than 0.5 mm in diameter. Yellow presumptively identified colonies are counted and confirmed by testing for the presence of cytochrome *c* (oxidase test) and the ability to ferment trehalose.

1.3 This method is designed to meet the monitoring requirements of the U.S. Environmental Protection Agency. *Aeromonas hydrophila* was included on the Contaminant Candidate List (CCL) (Mar. 2, 1998, 63 *FR* 10274) and in the Revisions to the Unregulated Contaminant Monitoring Proposed Rule (UCMR) (September 17, 1999, 64 FR 50556). Contaminants in the UCMR are candidates for future regulation and may be included in a monitoring program for unregulated contaminants. Unregulated contaminant monitoring would be required for large systems and a representative sample of small and medium sized water distribution systems.

1.4 EPA intends to conduct a multi-lab validation of this method in finished waters.

2.0 Summary of Method

2.1 The method provides a direct count of *Aeromonas* in water based on the development of yellow colonies on the surface of the membrane filter using a selective media for *Aeromonas* species. A water sample is filtered through 0.45-µm-pore-size membrane filter. The filter is placed on ampicillin-dextrin agar and incubated at 35°C ± 0.5°C for 22 to 26 hours. This medium uses 10 mg/L ampicillin to inhibit non-*Aeromonas* species, while allowing most *Aeromonas* to grow. The medium uses dextrin as a fermentable carbohydrate, and bromothymol blue as an indicator of acidity produced by the fermentation of dextrin. Yellow presumptively identified colonies are counted and confirmed by testing for the presence of cytochrome *c* (oxidase test) and the ability to ferment trehalose.

The membrane filtration procedure provides a direct count of culturable *Aeromonas* in water samples that is based on the growth of bacterial colonies on the surface of the membrane filter.

If samples are to be archived for further analysis to determine species or hybridization group, from the nutrient agar plate, either inoculate a nutrient agar slant for short term use or shipment to another laboratory or inoculate a tube of nutrient agar broth for internal storage in the freezer.

3.0 Definitions

3.1 *Aeromonas* are bacteria that are facultative anaerobes, Gram-negative, oxidase-positive, polarly flagellated, and rod shaped. They are classified as members of the family *Aeromonadaceae*. Demarta et al. (1999) reported 15 *Aeromonas* species based on 16S rDNA sequences though not all are officially recognized. Some, but not all, have been associated with human disease. In this method, *Aeromonas* are those bacteria that grow on ampicillin-dextrin agar (ADA), produce yellow colonies, are oxidase-positive, and ferment trehalose.

3.2 Definitions for other terms are provided in the glossary at the end of the method (Section 17.3).

4.0 Interferences and Contamination

4.1 This method is designed to be used with finished water. Water samples containing colloidal or suspended particulate material may clog the membrane filter and prevent filtration or cause spreading of bacterial colonies which could interfere with identification of target colonies.

4.2 Other ampicillin-resistant bacteria that are not aeromonads may be able to grow on this medium. Some of these bacteria may also produce yellow colonies if they are able to produce acid byproducts from the fermentation of dextrin or some other media component, or if they produce a yellow pigment. Enterococcus bacteria are reported to produce pinpoint-size yellow colonies on ADA. Confirmation of presumptive *Aeromonas* colonies is necessary to mitigate false positives.

5.0 Safety

5.1 Since some strains of *Aeromonas* are opportunistic pathogens, sample containers and waste materials should be autoclaved prior to cleaning or disposal.

5.2 The analyst/technician must know and observe the normal safety procedures required in a microbiology laboratory while preparing, using and disposing of cultures, reagents, and other materials.

5.3 This method does not address all safety issues associated with its use. The laboratory is responsible for maintaining a safe work environment and a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material safety data sheets (MSDSs) should be available to all personnel involved in these analyses.

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6.0 Equipment and Supplies

Note: *Brand names, suppliers, and part numbers are for illustrative purposes only. No endorsement is implied. Equivalent performance may be achieved using apparatus and materials other than those specified here, but demonstration of equivalent performance that meets the requirements of this method is the responsibility of the laboratory.*

6.1 Equipment for collection and transport of samples to laboratory

- **6.1.1** Autoclavable sample bottles—1-L glass or plastic, sterile, with sufficient head space for mixing sample
- **6.1.2** Ice chest
- **6.1.3** Ice packs

6.2 Autoclavable dilution bottles—125-mL marked at 99 mL or 90 mL; commercially produced dilution bottles may be used

6.3 Rinse water bottles

6.4 Sterile plastic or autoclavable glass pipettes with a 2.5% tolerance—To deliver (TD), 1- and 10-mL

6.5 Pipet bulbs or automatic pipetter

6.6 Autoclavable pipette container (if using glass pipettes)

6.7 Thermometer—with 0.5°C gradations checked against a National Institute of Standards and Technology (NIST) certified thermometer, or one that meets the requirements of NIST Monograph SP 250-23

6.8 Inoculating loop—Sterile metal, plastic, or wooden applicator sticks

6.9 Burner—Flame or electric incinerator for sterilizing metal inoculating loops and forceps

6.10 Colony counting device—Mechanical, electric or hand tally

6.11 Hotplate stirrer

6.12 Magnetic stir bar

6.13 Graduated cylinders—100 mL, 500 mL and 1 L, sterile, polypropylene or glass

6.14 Balance—Capable of weighing samples up to 200 g

6.15 Weigh boats

6.16 pH meter

6.17 Turbidimeter (optional)

6.18 Equipment for membrane filter procedure

- **6.18.1** Incubator—Hot air or water-jacketed microbiological type to maintain a temperature of 35°C ± 0.5°C
- **6.18.2** Petri dishes—sterile, 50 × 9 mm or other appropriate size

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6.18.3 Membrane filtration units (filter base and funnel made of glass, plastic, or stainless steel) autoclavable and wrapped with aluminum foil or Kraft paper and sterilized

6.18.4 Vacuum source

6.18.5 Flasks—1-L vacuum filter with appropriate tubing; a filter manifold to hold a number of units is optional

6.18.6 Side-arm flask to place between vacuum source and filtration devices or filter manifold

6.18.7 Membrane filters—Sterile, cellulose ester, white, gridded, 47-mm-diameter with 0.45-μm pore size

6.18.8 Forceps—Sterile, straight or curved, with smooth tips to handle filters without causing damage

6.18.9 Ethanol or other alcohol in a container to sterilize forceps

6.19 Dissecting microscope—Low power (10X to 15X), binocular, illuminated

6.20 Autoclave—Capable of 121°C at 15 psi. Must meet requirements set forth in the *Manual for the Certification of Laboratories Analyzing Drinking Water, 4th Edition.*

6.21 Membrane filters (for sterilization purposes)—Sterile, cellulose ester, with 0.22-μm pore size

7.0 Reagents and Standards

7.1 Purity of reagents and culture media—Reagent-grade chemicals shall be used in all tests. Unless otherwise indicated, reagents and culture media shall conform to the specifications in *Standard Methods for the Examination of Water and Wastewater* (latest edition approved by EPA in 40 CFR Part 141), Section 9050 (Reference 15.2). The agar used in preparation of culture media must be of microbiological grade.

7.2 Purity of water —Reagent-grade water conforming to specifications in *Manual for the Certification of Laboratories Analyzing Drinking Water, 4th Edition* or *Standard Methods for the Examination of Water and Wastewater* (latest edition approved by EPA in 40 CFR Part 141), Section 9020 (Reference 15.2).

7.3 Phosphate buffered dilution water

7.3.1 Concentrated stock phosphate buffer solution—Dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL reagent-grade water. Adjust the pH to 7.2 ± 0.5 with 1N sodium hydroxide (NaOH) and dilute to 1 L with reagent-grade water. Autoclave or filter sterilize through a filter with 0.22 μm pore size.

7.3.2 Magnesium chloride solution—Dissolve 81.1 g magnesium chloride hexahydrate ($MgCl_2 6H_20$) in reagent-grade water and dilute to 1 L.

7.3.3 Prepare phosphate buffered dilution water by adding 1.25 mL of concentrated stock phosphate buffer solution (Section 7.3.1) and 5.0 mL of magnesium chloride solution (Section 7.3.2) to a 1-L graduated cylinder and adjust final volume to 1 L with reagent-grade water. Prepare a portion of buffered dilution water in 1-L bottles for rinse water. Autoclave or filter sterilize through a filter with 0.22 μm pore size.

7.3.4 Phosphate buffered dilution water may be stored indefinitely, unless turbidity is observed.

7.4 Ampicillin-dextrin agar (ADA)— Tec Pac - Biolife Italiana cat. No. 401019 (M-Aeromonas Selective Agar Base [Havelaar]) Uile Monza, Milano, Italy., or equivalent. Follow procedure as specified on media for preparation. EPA highly recommends the use of commercial ADA., however ADA may be prepared as follows, if necessary.

7.4.1 5.0 g tryptose—Difco cat. no. 0124-17-2, or equivalent

7.4.2 10.0 g dextrin—Difco cat. no. 0161-17-6

7.4.3 2.0 g yeast extract—Difco cat. no. 0127-17-9, or equivalent

7.4.4 3.0 g sodium chloride (NaCl)—Baker cat. no. 3624-01, or equivalent

7.4.5 2.0 g potassium chloride (KCl)—Fisher cat. no. P217-500, or equivalent

7.4.6 0.2 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)—Fisher cat. no. M63-500, or equivalent

7.4.7 0.1 g iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$)—Sigma cat. no. F-2877, or equivalent

7.4.8 0.08 g bromothymol blue—Baker cat. no. 1-D470, or equivalent

7.4.9 15 g agar, bacteriological grade—Fisher cat. no. BP1423-500, or equivalent

7.4.10 Ampicillin, sodium salt—Sigma A0166, or equivalent. Add 10 mg of ampicillin, sodium salt to 10 mL reagent water. Prepare on the same day that media is prepared and filter sterilize through a filter with 0.22 μm pore size. Follow manufacturer's instructions for appropriate storage temperature and length.

7.4.11 Sodium deoxycholate—Sigma cat. no. D-6750, or equivalent. Add 100 mg of sodium deoxycholate to 10 mL of reagent water. Filter sterilize through a filter with 0.22 μm pore size.

7.4.12 Add reagents in Sections 7.4.1 through 7.4.9 to 1-L of reagent-grade water, cool to room temperature, and adjust pH to 8.0 using 1N NaOH or 1N HCl. Autoclave for 15 min, cool to 50°C, and add the sterile ampicillin and sterile sodium deoxycholate solutions.

Note: *The agar (Section 7.4.9) may be added after the pH is adjusted, if it is more convenient for the laboratory. Agar must be dissolved before the media is autoclaved.*

7.4.13 Add approximately 5mL of ADA per 50 × 9 mm petri dish and allow to solidify. For larger plates, adjust volume appropriately. ADA plates should be stored in a tight fitting container (i.e. sealed plastic bag) at a temperature of 1°C to 5°C for no longer than 14 days.

7.5 Pentahydrate ACS Reagent grade sodium thiosulfate—Fisher cat. no. S446, or equivalent.

7.5.1 Stock solution (3 % solution)—Add 3 g sodium thiosulfate to 100 mL reagent-grade water.

7.5.2 Add 1 mL of sodium thiosulfate per L of sample to sample bottles prior to autoclave sterilization. Alternatively, if using presterilized sample bottles, sodium thiosulfate should be autoclaved for 15 minutes or filter sterilized through a filter with 0.22 μm pore size before adding to the sample bottles.

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7.6 Disodium salt of ethylenediaminetetraacetic acid (EDTA)—Sigma cat. no. E 4884, or equivalent. EDTA should only be added to samples, if metals in water samples exceed 1.0 mg/L.

7.6.1 Stock solution—Add 372 mg EDTA to 1 L of reagent-grade water.

7.6.2 Working solution (15% solution)—Add 15 mL of the stock solution to 100 mL of reagent grade water and adjust pH to 6.5 using 1N NaOH or 1N HCl before sterilization.

7.6.3 If EDTA is necessary to reduce metal toxicity, add 2.5 mL of the working solution per L of sample to sample bottles prior to autoclave sterilization. If using presterilized sample bottles, EDTA should be autoclaved for 15 minutes or filter sterilized through a filter with 0.22 μm pore size.

7.7 Positive control culture—*Aeromonas hydrophila* ATCC #7966; obtained from the American Type Culture Collection (ATCC, 10801 University Blvd, Manassas, VA, 20110-2209).

7.8 Negative control—One or more non-*Aeromonas* bacteria which grow on ADA will be identified to be used as negative culture controls. The purpose of these is to help the analyst recognize other bacteria which may grow on ADA.

7.9 Nutrient agar —Difco cat. no. 0001-17-0 or equivalent

7.10 Oxidase reagents—Sigma cat. no. T3134, or Dry Slide - Oxidase Disposable Slide Tests (Difco DF3530-75-3) or BBL Reagent Droppers (0.5mL) B-D 4361181 or equivalent

7.11 0.5% Trehalose confirmation reagent - add 5 g trehalose (Sigma cat. no. T0167, or equivalent) to 100 mL water and filter sterilize solution. Prepare purple broth base (Difco cat. no. 222710) according to manufacturer directions as for one liter but only use 900 mL water. Autoclave purple broth base. Cool to room temperature. Aseptically, add trehalose solution to cooled purple broth base. Store in refrigerator or preferably, prepare less adjusting proportions accordingly. Dispense into 6mL or larger size tubes and fill approximately half full.

8.0 Sample Collection, Preservation, and Storage

8.1 Use 1-L glass or plastic bottles (Section 6.1.1). Sampling procedures are described in detail in *Standard Methods for the Examination of Water and Wastewater*, Section 9060 (Reference 15.2). Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Sample results will be considered invalid if those conditions are not met.

8.2 Sample collection—Samples must be representative of the drinking water distribution system. Water taps used for sampling should be free of aerators, strainers, hose attachments, mixing type faucets, and purification devices. Cold water taps should be used. The service line should be cleared before sampling by maintaining a steady water flow for at least two minutes (until the water changes temperature).

8.2.1 Add 1 mL of sodium thiosulfate per L of sample (Section 7.5).

8.2.2 If metals in the sample exceed 1.0mg/L, add 2.5 mL of EDTA working solution per L of sample to reduce metal toxicity (Section 7.6).

8.2.3 Collect a minimum of 1-L of sample in a sterile, non-toxic 1-L glass or plastic container with a leak-proof lid. Leave headspace to allow mixing.

8.3 Sample preservation and handling

8.3.1 Immediately following sample collection, tighten the sample container lid(s) and place the sample container(s) in an insulated storage cooler with ice packs or in a refrigerator to chill prior to packing the cooler for shipment. Do not freeze the sample.

8.3.2 Place the chilled sample(s) upright in the center of a plastic-lined, insulated container. Use insulated containers to ensure proper maintenance and storage temperature. Use enough freshly frozen ice packs to ensure that the samples will arrive at a temperature of 1°C to 10°C. Use a minimum of two ice packs per shipment and add extra ice packs for multiple samples. Place one or more ice packs on each side of the container to stabilize samples.

8.3.3 Samples must be maintained at a temperature of 1°C to 10°C during shipment. Samples must not be frozen.

Note: *Sample temperature during shipment is critical. Ice packs must be "fresh" (frozen solid) immediately prior to shipment.*

8.4 Refrigerate samples at 1°C to 5°C upon receipt at the laboratory and analyze as soon as possible after collection. Samples must be analyzed within 30 hours of sample collection. Holding time study results are summarized in Section 16, Table 2.

9.0 Quality Control

Note: *The quality control requirements in Section 9 are based on the existing* U.S. EPA *Manual for the Certification of Laboratories Analyzing Drinking Water, Fourth Edition, the analytical quality control requirements required in the Unregulated Contaminant Monitoring Rule, and comments during review of the April 2000 version of this method. These requirements are subject to change after evaluation of the interlaboratory validation results.*

9.1 Each laboratory that uses this method is required to operate a formal quality assurance (QA) program. The minimum requirements of a quality control program for this method consist of initial and ongoing demonstrations of laboratory capability through analysis of positive and negative control samples and method blanks. Laboratory performance is compared to the performance criteria specified in Section 9.4 to determine whether the results of the analyses meet the performance characteristics of the method.

9.2 Specific quality control (QC) requirements for Method 1605 are provided below. QA and QC criteria for facilities, personnel, and laboratory equipment, instrumentation, and supplies used in microbiological analyses must be followed according to *Standard Methods for the Examination of Water and Wastewater* (latest edition approved by EPA in 40 CFR Part 141) and the U.S. EPA

Manual for the Certification of Laboratories Analyzing Drinking Water, Fourth Edition (March 1997) (Reference 15.5).

9.3 Dilution scheme for determining organism density. This procedure is adapted from *Standard Methods for the Examination of Water and Wastewater, 19th Edition*, Section 9020 B (Reference 15.9). This entire process should be performed quickly to avoid loss of viable organisms. See Section 16, Flowchart 1, for an example of this dilution scheme.

9.3.1 Inoculate organisms onto the entire surface of several nutrient agar slants with a slope approximately 6.3 cm long in a 125 × 6 mm screw-cap tube. Incubate for 18 to 24 hours at 35°C.

9.3.2 From the slant which has the best growth, prepare serial dilutions using four dilution bottles with 99 mL of sterile buffered dilution water (A, B, C and D below) and one dilution bottle containing 90-mL of sterile buffered dilution water (D2 below).

9.3.3 Pipet 1 mL of buffered dilution water from bottle "A" to one of the slants. Emulsify the growth on the slant by gently rubbing the bacterial film with the pipet, being careful not to tear the agar. Pipet the suspension back into dilution bottle "A.". Repeat this procedure a second time to remove any remaining growth on the agar slant, without disturbing the agar.

9.3.4 Make serial dilutions as follows:

9.3.4.1 Shake bottle "A" vigorously and pipette 1 mL to bottle "B".

9.3.4.2 Shake bottle "B" vigorously and pipette 1 mL to bottle "C".

9.3.4.3 Shake bottle "C" vigorously and pipette 1 mL to bottle "D".

9.3.4.4 Shake bottle "D" vigorously and pipette 10 mL to bottle "D2"; this should result in a final dilution of approximately 10 CFU / mL.

9.3.5 Filter 1- to 5-mL portions in triplicate from bottles "D" and "D2" according to the procedure in Section 10 to determine the number of CFU in the dilutions. The target dilution is one that produces 20 to 80 colonies per ADA plate. Dilutions should be stored at 1°C to 5°C and may be used throughout the day they are prepared.

Note: *Analysts may practice the dilution scheme by placing filters on nutrient agar plates instead of ADA plates. After a growth pattern is determined and the analyst can accurately determine the target concentrations, dilutions from Section 9.3.5 may be filtered in duplicate. However, dilutions should be analyzed in triplicate when new cultures are used.*

9.3.6 There should be approximately 10^{10} *Aeromonas hydrophila* CFU per slant. Therefore, dilution bottles "A" through "D2" should contain approximately 10^{10}, 10^{8}, 10^{6}, 10^{4}, and 10^{3} CFU per dilution bottle, respectively.

Note: Depending on the growing conditions and the strain and species selected, these numbers may vary. As a result, until experience has been gained with the conditions and organisms used, more dilutions may need to be filtered to determine the appropriate dilution.

9.4 Analytical QC for the membrane filter (MF) procedure. The laboratory must successfully analyze a positive control sample (Section 9.4.1), negative control sample (Section 9.4.2), and method blank (Section 9.4.3) before performing any field sample analyses using this method.

9.4.1 Positive control and positive control duplicate—this must be performed once per week that samples are analyzed or once per media batch, whichever is more frequent.

9.4.1.1 Using a pure culture obtained from a qualified outside source (Section 7.7), grow *Aeromonas* on a 24 hour nutrient agar slant.

9.4.1.2 Prepare a stock culture and dilute to a density of approximately 10 CFU per mL by the procedure listed in Section 9.3. For each (positive control and positive control duplicate), spike enough volume of the appropriate dilution into 500 mL of reagent water to obtain 20-80 CFU per filter. Filter immediately after spiking. Process positive control and positive control duplicate according to the procedure in Section 10. Confirm positive control and positive control duplicate by the procedure listed in Section 10.11.

9.4.1.3 Calculate the relative percent difference (RPD) using the following equation:

$$RPD = 100 \times \frac{|(PC) - (PCD)|}{[(PC + PCD) / 2]}$$

where

RPD is the relative percent difference
PC is the density of *Aeromonas* in the positive control sample (CFU / 100 mL)
PCD is the density of *Aeromonas* in the positive control duplicate sample (CFU / 100 mL)

9.4.1.4 The percent difference between duplicate positive controls should not exceed [to be determined through method validation].

9.4.1.5 If target colonies do not appear on ADA agar, or do not confirmed, ***halt all sample analyses.*** Prepare new media and culture dilutions and analyze a second positive control sample. If results are still unacceptable, then culture viability or reagent potency may have been compromised. To identify whether a problem is due to the positive control rather than the ADA agar,

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it may be advisable to filter an extra positive control in 9.4.1.2 and place the filter on a nutrient agar plate.

9.4.2 Negative control—On an ongoing basis, the laboratory must perform, at a minimum, one negative control per media batch.

9.4.2.1 Using pure cultures obtained from a qualified outside source (Section 7.8), grow negative control cultures on a 24 hour nutrient agar slant.

9.4.2.2 Streak negative controls onto ADA agar to become familiar with the color and morphology of non-*Aeromonas* bacteria.

9.4.3 Method blank—On an ongoing basis, dilution/rinse water method blanks must be processed at the beginning and end of each filtration series to check for possible cross-contamination. A filtration series ends when 30 minutes or more elapse between sample filtrations. An additional method blank is also required for every 20 samples, if more than 20 samples are processed during a filtration series. For example, if a laboratory plans to run 30 samples during a filtration series, a method blank should be processed at the beginning, middle, and end of the filtration series.

9.4.3.1 Process 100-mL dilution/rinse water method blanks according to the procedures in Section 10, as appropriate.

9.4.3.2 No growth should appear in method blanks. If growth appears, ***halt all analyses***. Prepare new dilution/rinse water and reanalyze the method blanks. If colonies are present after analyzing the new dilution/rinse water, assess laboratory technique and reagents.

9.5 A tube of the 0.5% trehalose (Section 7.11) should be incubated with every batch of samples to confirm sterility.

9.6 If the laboratory has two or more analysts, each are required to count target colonies on the same membrane from one positive sample per month. Compare each analyst's count of the target colonies. Counts should fall within 10% between analysts. If counts fail to fall within 10% of each other, analysts should continue to perform counts, until the number of target colonies counted fall within 10% between analysts for at least three consecutive samples. If there are no positive samples, the positive control can be used for this determination.

9.7 Verify autoclave sterilization monthly by placing *Bacillus stearothermophilus* spore suspensions or strips inside glassware. Sterilize at 121 °C for 15 minutes. Place in trypticase soy broth tubes and incubate at 55°C for 48 hours. Check for growth to verify that sterilization was adequate. If sterilization was inadequate, determine appropriate time for autoclave sterilization. Filter sterilization may be used as long as the same quality controls are instituted for the filtrate.

9.8 Participate in interlaboratory performance studies conducted by local, state, and federal agencies or commercial organizations, if available.

10.0 Procedure

10.1 The membrane filter (MF) procedure with ampicillin-dextrin agar (ADA) is used to enumerate *Aeromonas* in finished waters.

10.2 Label each petri dish with sample identification, preparation date, and analysis start date/time.

10.3 Use a sterile MF unit assembly (Section 6.18.3) at the beginning of each filtration series. A filtration series ends when 30 minutes or more elapse between sample filtrations.

10.4 Sterilize forceps with alcohol. Flame off excess alcohol. Alternatively, an electric incinerator may be used to sterilize forceps (Section 6.9). Using sterile forceps, place the MF (grid side up) over the sterilized funnel. Carefully place the top half of the filtration unit over the funnel and lock it in place.

10.5 Shake the sample bottle vigorously approximately 25 times to distribute the bacteria uniformly. Using aseptic technique, transfer one, 500-mL aliquot of sample to a single funnel. Use a graduated cylinder with a "to deliver" tolerance of approximately 2.5%.

Note: *Laboratories must filter the entire 500-mL sample volume unless the filter clogs. If the filter clogs, a minimum of 100 mL of sample must be filtered, which may require multiple filtrations. If less than 500 mL are filtered and analyzed due to filter clogging, measure the residual, unfiltered volume to determine the volume filtered, and adjust the reporting limit accordingly.*

10.6 Filter each sample under partial vacuum (10-15 mmHg) through a sterile membrane filter. Rinse the funnel after each sample filtration by filtering three, 20-mL to 30-mL portions of sterile buffered dilution water.

10.7 Upon completion of the final rinse, disengage the vacuum and remove the funnel.

10.8 Using sterile forceps, immediately remove the MF and place it grid-side-up on the ADA medium with a rolling motion to avoid trapping air under the filter. Reseat the membrane filter if bubbles occur. Place the inverted petri dishes in the 35°C ± 0.5°C incubator immediately after preparation. Sterilize forceps and filtering apparatus between the preparation of each sample.

10.9 After 22 to 26 hours of incubation at 35°C ± 0.5°C, count and record yellow colonies with a diameter greater than 0.5 mm under magnification using a dissecting microscope.

10.10 *Aeromonas* confirmation—Isolation of a yellow colony with a diameter greater than 0.5 mm on ampicillin-dextrin agar (ADA) should be considered a presumptive positive for *Aeromonas*. All presumptive *Aeromonas* colonies up to ten per sample must be tested for the presence of cytochrome *c* (oxidase test - Section 10.10.1). Colonies positive for the oxidase test must be tested for the ability to ferment trehalose (Section 10.10.3). In this method, any presumptive colony that is positive for oxidase and ferments trehalose is considered to be *Aeromonas*. Slight variations in color and morphology may be present between different *Aeromonas* species grown on ADA medium. The colonies selected for confirmation should be representative of all the presumptively positive colonies grown on the ADA plate. For example, if 30 bright yellow colonies and 20 dull yellow colonies are observed, then 6 bright yellow and 4 dull yellow colonies should be submitted to confirmation.

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Note: *It is important to record the number of colonies of each presumptively positive morphological type so that the final density of Aeromonas can be reported based on percent confirmation of each morphological type. Also, the laboratory may submit more than ten presumptively positive colonies to the confirmation step.*

10.10.1 To confirm as *Aeromonas*, pick a colony and streak the colony onto a plate of nutrient agar medium (Section 7.9) and incubate at 35°C ± 0.5°C for 22 to 26 hours to obtain isolated colonies.

Note: *If the streak plate has more than one morphological type of colony, submit one of each type to the confirmation steps.*

10.10.2 The oxidase test can be performed with a freshly made solution or a commercially available preparation of N,N,N,N-tetramethyl-p-phenylenediamine (1%, aqueous) (Section 7.10). If using a freshly made solution, saturate the pad. Apply a small amount of a discreet colony from the nutrient agar to the oxidase pad using a wooden or plastic applicator. A blue/purple color reaction within 10 seconds is considered a positive oxidase test.

Note: *Timing of the color reaction is critical, as some Gram-positive bacteria may give false positives after 10 seconds. Also, it is important to put just a small amount of the colony on the oxidase. Too much bacteria can also cause a false positive oxidase test.*

10.10.3 If the oxidase test is positive, then test for trehalose fermentation. Trehalose fermentation is determined by inoculating a tube containing 3-10 mL (depending on the size of the tube used - fill about half full) of 0.5% trehalose in purple broth base (Section 7.11) with a colony from the nutrient agar and incubating at 35°C for 24 ± 2 hours. A change in color of the medium from purple to yellow is considered a positive for trehalose fermentation.

10.10.4 If a colony is both oxidase and trehalose positive, report as a confirmed *Aeromonas* and archive the colony for further identification. As part of the UCMR monitoring, this further identification will be performed by EPA.

Note: *If samples are to be archived for further analysis to determine species or hybridization group, from the nutrient agar plate (Section 10.10.1), either inoculate a nutrient agar slant for short term use or shipment to another laboratory or inoculate a tube of nutrient agar broth for internal storage in the freezer.*

11.0 Data Analysis and Calculations

11.1 See *Standard Methods for the Examination of Water and Wastewater* (Reference 15.2) for general counting rules. The density of *Aeromonas* determined by the membrane filter (MF) procedure is calculated by direct identification and enumeration of yellow colonies by a dissecting microscope (Section 6.19) followed by oxidase and trehalose confirmation. Bacterial density is recorded as presumptive *Aeromonas* colony forming units (CFU) per 100 mL of sample and confirmed *Aeromonas* CFU per 100 mL.

11.2 Counting colonies on ADA

11.2.1 Record the number of presumptive *Aeromonas* CFU/100mL. If there is more than one morphological type that is considered to be presumptively positive, record the number of presumptive positives for each morphological type, as well as the total number of presumptive positives.

11.2.2 If there are more than 200 colonies, including background colonies, report results as too numerous to count (TNTC) and resample. If resampling is necessary, an undiluted 500-mL sample and a minimum of three dilutions should be analyzed.

11.2.3 If the colonies are not discrete and appear to be growing together, report results as confluent growth (CNFG) and resample.

11.3 Confirmation and calculation of *Aeromonas* density

11.3.1 All presumptive colonies that are oxidase positive and ferment trehalose are confirmed as *Aeromonas*. For the final density of confirmed *Aeromonas*, adjust the initial, presumptive count based on the positive confirmation percentage for each presumptively positive morphological type and report as confirmed CFU per 100 mL.

11.3.2 Calculate the number of positive confirmations for ***each*** presumptively positive morphological type ***from all filters of a given sample*** using the following equation:

$$\left(\frac{\text{No. positively confirmed}}{\text{No. submitted to confirmation}} \times \text{No. presumptive positives}\right) \times \frac{100}{\text{No. mL filtered}} = \text{Confirmed Aeromonas / 100mL}$$

11.3.3 Record the number of confirmed *Aeromonas* per 100 mL for each colony morphology.

11.3.4 Sum the number of confirmed *Aeromonas* per 100 mL for all presumptively positive colony types (Section 11.3.2) and report as the density of confirmed *Aeromonas* per 100 mL.

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11.3.5 **Example 1:** In this example, ***500 mL*** of sample was filtered and two different morphological types of presumptively positive colonies were observed.

Example 1

Morphological Description	No. presumptive positive colonies	No. submitted to confirmation steps	No. positively confirmed	No. of confirmed *Aeromonas* per 100 mL
Type A: Bright yellow, round, opaque	30	6	6	6
Type B: Dull yellow, oval, translucent	20	4	3	3
Total number of confirmed *Aeromonas* per sample:				**9 per 100 mL**

$$\left(\frac{6}{6} \times 30\right) \times \frac{100}{500} = 6 \text{ Confirmed Type A Aeromonas / 100mL}$$

$$\left(\frac{3}{4} \times 20\right) \times \frac{100}{500} = 3 \text{ Confirmed Type B Aeromonas / 100mL}$$

Example 1 results in 9 confirmed *Aeromonas* / 100 mL.

11.3.6 **Example 2:** In this example, ***200 mL*** of sample was filtered and two different morphological types of presumptively positive colonies were observed.

Example 2

Morphological Description	No. presumptive positive colonies	No. submitted to confirmation steps	No. positively confirmed	No. of confirmed *Aeromonas* per 100 mL
Type A: Dull yellow, round, opaque	40	5	5	20
Type B: Dull yellow, round, translucent	40	5	3	12
Total number of confirmed *Aeromonas* per sample:				**32 per 100 mL**

$$\left(\frac{5}{5} \times 40\right) \times \frac{100}{200} = 20 \text{ Confirmed Type A Aeromonas / 100mL}$$

$$\left(\frac{3}{5} \times 40\right) \times \frac{100}{200} = 12 \text{ Confirmed Type B Aeromonas / 100mL}$$

Example 2 results in 32 confirmed *Aeromonas* / 100 mL.

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11.3.7 If there were no presumptively positive colonies or if none of the presumptive colonies are confirmed, then report the results as less than the detection limit (DL) in CFU per 100 mL based on sample volume filtered. If less than 500 mL are filtered, then adjust the reporting limit per 100 mL accordingly. The DL may be calculated as follows:

DL per 100 mL = 100 / volume filtered CFU per 100mL

11.3.7.1 **Example 3:** If 500 mL of sample was filtered end there were no confirmed colonies, then report as <0.2 CFU/100 mL.

11.3.7.2 **Example 4:** If 100 mL of sample was filtered and there were no confirmed colonies, then report as <1.0 CFU/100 mL.

12.0 Method Performance

12.1 Specificity of media

12.1.1 Of the 30 *Aeromonas* strains tested, 21 grew will on ADA at 35°.

12.1.2 ADA was able to support the growth of all the species most often associated with human disease.

12.1.3 Efforts continue to identify colonies which give a presumptive positive on the ADA media but do not confirm.

12.2 Bias: To be determined through method validation

12.3 Precision: To be determined through method validation

12.4 Method performance: To be determined through method validation

13.0 Pollution Prevention

13.1 The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly.

13.2 Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be disposed.

14.0 Waste Management

14.1 It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the biohazard waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required.

14.2 Samples, reference materials, and equipment known or suspected of having bacterial contamination from this work must be sterilized prior to disposal.

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14.3 For further information on waste management, consult "The Waste Management Manual for Laboratory Personnel" and "Less is Better: Laboratory Chemical Management for Waste Reduction", both of which are available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington, D.C. 20036.

15.0 References

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16.0 Tables, Diagrams, Flowcharts, and Validation Data

Table 1. Growth of ATCC cultures on ADA at 30° and 35°C in 24 hrs

Collection #	Hybridization group	*Aeromonas* species	Growth at 30°C	Growth at 35°C
ATCC 7966	Group 1	*hydrophila*	+	+[1]
ATCC 35654	Group 1	*hydrophila*	+	+[1]
AMC 12723-W	Group 1	*hydrophila*	+	+[1]
ATCC 51108	Group 2	*bestiarum*	+	+[1]
AMC 14228-V	Group 2	*bestiarum*	+	+[1]
ATCC 33658[2]	Group 3	*salmonicida/salmonicida*	-	-
AMC 15228-V	Group 3	*salmonicida*	+	+
ATCC 15468	Group 4	*caviae*	+	+[1]
MML 1685-E	Group 4	*caviae*	+	+[1]
ATCC 33907	Group 5	*media*	-	-
AMC Leftwich	Group 5	*media*	-	-
ATCC 23309[2]	Group 6	*eucrenophila*	+	-
ATCC 35993	Group 7	*sobria*	+	+
Muldoon SMHC	Group 7	*sobria*	+	+[1]
ATCC 9071	Group 8	*veronii/sobria*	+	+[1]
AMC 1123-W	Group 8	*veronii/sobria*	+	+
ATCC 43700[3]	Group 12	*schubertii*	+	+
AMC 1108-W	Group 12	*schubertii*	+	-
ATCC 49657[4]	-	*trota*	-	-
NMRI 206	-	*trota*	-	-
ATCC 51208		*allosaccharophila*	+	+
ATCC 49568	Group 9	*jandaei*	+	+
ATCC 49569	Group 9	*jandaei*	+	+
ATCC 35622	Group 10	*veronii/veronii*	+	+
WR4659	Group 10	*veronii/veronii*	+	+
CECT 4342	Group 11	*encheleia*	+	-
LMG 17541[5]	-	*popoffii*	+	+[1]
AMC (ATCC 35941)	-	ornithine positive	-	-

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Collection #	Hybridization group	*Aeromonas* species	Growth at 30°C	Growth at 35°C
AMC (ATCC 43946)	-	Group 501	+	+[1]
CDC 0434-84	Group 3	Motile Group 3	+	+[1]

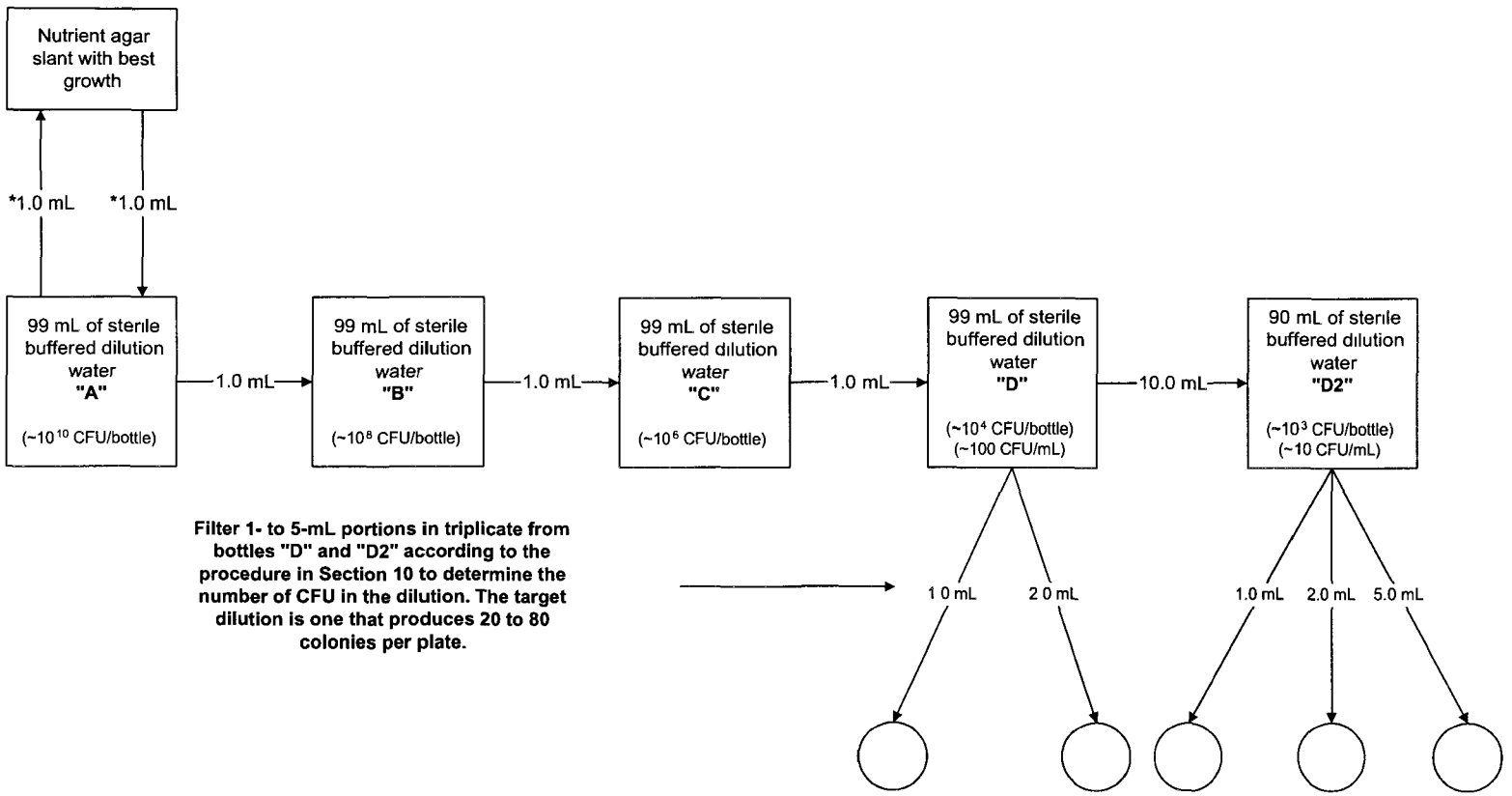
(1) Organisms displayed brighter yellow color and/or were larger on ADA media at 35°C
(2) Respective *Aeromonas* cultures grew on ADA medium at 35°C when streaked, but not when filtered.
(3) Respective *Aeromonas* cultures grew when streaked on ADA medium, however filtration was not performed with these cultures.
(4) Respective *Aeromonas* cultures did not grow on ADA medium when streaked.
(5) Respective *Aeromonas* cultures grew poorly on ADA medium at both temperatures.
Data for empty cells will be incorporated once analyses at different temperatures are complete.
ATCC = American Type Culture Collection, Rockville, MD. Other cultures were obtained from Amy Carnahan, University of Maryland. The wild type was obtained from Gene Rice, U.S. EPA. All cultures were grown on a slant, and transferred to 100 mL of buffered dilution water. Serial dilutions representing approximately 10-200 CFU were filtered and the membrane placed on ADA medium as described in Section 10. Additional membranes representing the same dilution for each of the respective cultures were placed on brain heart infusion agar as a control. All organisms tested oxidase and trehalose positive except the ATCC 35941 ornithine positive culture from Amy Carnahan.

Table 2. *Aeromonas* recovery based on holding time and the addition of EDTA

Species	Preservation State	% Recovery			
		Hour 0	Hour 6	Hour 24	Hour 30
A. caviae	w/o EDTA	100	79	34	24
	w/ EDTA	100	143	100	88
A. hydrophila	w/o EDTA	100	67	32	28
	w/ EDTA	100	107	86	92
A. veronii/sobria	w/o EDTA	100	92	58	43
	w/ EDTA	100	50	52	58
A. bestiarum	w/o EDTA	100	120	105	82
	w/ EDTA	100	120	302	285

For the holding time study conducted for this method, all samples were stored at 10°C. Results were compared using ADA media stored with the preservative EDTA with those held with no preservative. Four species of *Aeromonas* were analyzed: *A. caviae*, *A. hydrophila*, *A. veronii/sobria* and *A. bestiarum*. Results were similar for *A. caviae* and *A. hydrophila*, indicating that with no preservative there was a slight drop at 6 hours and a significant drop at 24 and 30 hours. While those preserved with EDTA remained fairly stable throughout the 30 hour holding time. However for *A. veronii/sobria* the sample preserved with EDTA saw a 50% loss by Hour 6, and then remained stable (at the 50% loss) until Hour 30. In contrast, the non-preserved sample stayed fairly stable for the first 6 hours then exhibited a 50% loss after one day. The density of unpreserved *A. bestiarum* remained fairly stable, while *A. bestiarum* preserved with EDTA showed increased densities.

Flowchart 1. Example Dilution And Inoculation Scheme For Determining Organism Density (Section 9.3)



***Pipet 1 mL of buffered dilution water from bottle "A" to the nutrient agar slant with the best growth. Emulsify the growth on the slant by gently rubbing the bacterial film with the pipet, being careful not to tear the agar, and pipet the solution back into bottle "A". Repeat this procedure a second time to remove any growth remaining on the agar slant.**

17.0 Glossary

17.1 Symbols

°C	degrees Celsius
μm	micrometer
±	plus or minus
<	less than
%	percent

17.2 Alphabetical characters and acronyms

ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection
CFR	Code of Federal Regulations
EDTA	ethylenediaminetetraacetic acid
g	gram
L	liter
mg	milligram
mL	milliliter
mm	millimeter
$Na_2S_2O_3$	sodium thiosulfate
NIST	National Institute of Standards and Technology
OSHA	Occupational Safety and Health Administration
psi	pounds per square inch
QC	quality control
TNTC	too numerous to count
USEPA	United States Environmental Protection Agency
X	"times"

17.3 Definitions

Method blank —A 100-mL aliquot of dilution/rinse water that is treated exactly as a sample and carried through all portions of the procedure until determined to be negative or positive. The method blank is used to determine if the sample has become contaminated by the introduction of a foreign microorganism through poor technique.

Must—This action, activity, or procedural step is required.

Negative control—A non-*Aeromonas* bacteria that is streaked onto ADA agar so that the analyst can become familiar with the color and morphology of non-*Aeromonas* bacteria.

Positive control—A 500-mL reagent water spiked with 20 - 80 CFU of *Aeromonas*. The positive control is analyzed exactly like a sample. Its purpose is to assure that the results produced by the laboratory remain within the limits specified in this method for precision and recovery.

Selective medium—A culture medium designed to suppress the growth of unwanted microorganisms and encourage the growth of the target bacteria.

Should—This action, activity, or procedural step is suggested but not required.

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Presumptive positive colonies—*Aeromonas* spp. that grow as yellow colonies on ampicillin-dextrin agar.

Confirmed colonies—Presumptively positive colonies that test positive for oxidase and ferment trehalose.

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